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The Estimation of Vitamin B-Complex as an Index of Nutritional Status.

James A. McKirdy.

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The Estimation of Vitamin B-Complex as an Index of Nutritional Status.

A Thesis

submitted in conformity with the requirements
for the degree of Master of Science by the
University of Alberta.

By

James Alexander McKirdy.

Department of Biochemistry
University of Alberta.
April, 1942.

Thesis
1942
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INTRODUCTION

The vitamin B-complex is composed of several factors which occur together, though perhaps in different proportions in different natural products. Some of these factors are definitely established such as thiamine hydrochloride (vitamin B_1), riboflavin (B_2), pyridoxine (B_6), pantothenic acid, and nicotinic acid. Other provisional factors include biotin, inositol, choline and p-aminobenzoic acid.

Small amounts of these factors are essential for normal health. Lack of these factors gives rise to certain pathological conditions. Thiamine deficiency manifests itself as beri-beri, an atrophic and degenerative neuritis. Ariboflavinosis is characterized by cheilosis and circumcorneal and conjuctival vascularization. Pellagra or nicotinic acid deficiency is accompanied by glossitis, stomatitis and a dermatitis characterized by pigmentation and keratosis, with occasional vesicles and bullae.

More frequently do deficiencies in more than one factor occur. The best treatment then consists in giving the whole natural vitamin B-complex. Sources of the B-complex used in making therapeutic preparations include yeast, liver, and rice bran. Frequently also the natural products from the above sources are "fortified" especially with thiamine and nicotinic acid.

The factors of the vitamin B-complex are water-soluble and are not stored to any great extent in the body with the possible exception of the liver. Normally then,

any surplus over the body's requirement is excreted, chiefly by way of the urinary tract.

Three methods can be used to determine an individual's nutritional status: (a) estimation of the adequacy of the vitamin in the diet; (b) estimation of the vitamin content of the urine; and (c) estimation of the degree of excretion of a test dose of the vitamin.

The first of these methods requires a knowledge of the individual's diet and food analysis tables. For the second it is necessary to know the average normal level of excretion. This latter is difficult to define due to insufficient data. The third method is not handicapped by any of these prerequisites.

Scope of Investigation

PART I deals with the estimation of thiamine (vitamin B_1).

PART II deals with a biological assay of certain vitamin B-complex preparations.

PART I

Three methods have been studied, a microbiological one and two chemical methods, for the assay of vitamin B_1 (thiamine).

1. Microbiological Method:

This method makes use of the organism Propionibacterium pentosaceum. Tatum et al. (14) showed in the fifth of a series of papers on "Growth Factors for Bacteria" that vitamin B_1 was a growth stimulant for the propionic acid-bacteria. Silverman and Werkman (12) showed that the addition of vitamin B_1 to cells of Propionibacterium pentosaceum (49W) grown on a B_1-deficient basal medium, resulted in an almost immediate increase in their anaerobic pyruvate metabolism as measured by the evolution of CO_2 in the Warburg manometric apparatus. This observation was used by the same authors (13) as the basis for a proposed method of assay of vitamin B_1.

Materials and Methods.

1. Source of Culture.

A culture of Propionibacterium pentosaceum (49W) was received on a medium composed of yeast extract, 0.5%; sodium lactate, 2%; glucose, 0.1%; pH = 6.8.

2. Media Used.

(a) Stock culture medium (13) consisting of glucose 1%, yeast extract (Bacto) 0.3%, peptone (Bacto) 0.2%.

As no Bacto yeast extract was immediately available, dry whole brewer's yeast (Type 188) was used in an attempt to

obtain the yeast extract factors. This gave an undesirable turbid medium in which cell growth did not show up so readily. Nevertheless, 25 ml. of this medium was prepared and inoculated from the original tube. Transfer was made every other day, 4 to 5 drops of inoculum being added to 25 ml. of the same medium. Later a watery extract of the yeast was used in place of the whole yeast. This gave a fairly clear medium which supported bacterial growth but only about one-half as well as Bacto yeast extract which was finally employed.

(b) Basal medium of Tatum et al. (14), supplemented with cystine and hydrolyzed casein according to Silverman and Werkman consists of the following: glucose 1%, sodium acetate 0.6%, ammonium sulphate 0.3%, cystine 0.0050%, hydrolyzed casein 0.075%, ether extract of 3 gm. yeast extract per 100 ml. of medium, Speakman's salts in half concentration.

No reference was made in the work of Silverman and Werkman (13) as to how the hydrolyzed casein was prepared. Two methods of preparation were used here. Seventy-five grams of commercial casein was washed ten times with 0.1% acetic acid in order to free it of vitamin B_1, once with 60% ethanol and once with 95% ethanol, then air-dried in shallow pans. A portion of this washed casein (18.75 gm.) was digested with pancreatin according to Kahn as quoted by Levine and Schoenlein (7). Another portion (18.75 gm.) of washed casein was hydrolyzed with hydrochloric acid according to Gladstone and Fildes (2).

Ether extract of yeast extract was prepared from Bacto yeast extract according to Wood et al. (16). The ether extraction was carried out in a battery of six Soxhlet units.

Stock solutions of glucose, sodium acetate, ammonium sulphate and Speakman's salts (16) were prepared.

(c) Plating medium was that used by Werkman and Brown (15) and consists of yeast extract 1%, dipotassium phosphate 0.1%, agar 1.5%, together with glucose 1%.

(d) Cultural Reaction Media. -- The plating medium without agar was used as base, the various carbohydrates, arabinose, dextrin, glucose, starch, sucrose, xylose, being added in turn in concentration of 1%.

3. Staining Procedure.

The Hucker modification of the Gram stain was used.

4. Method of Purification of Bacteria.

Poured plates were inoculated from the stock culture. Colonies were picked into liquid medium and subjected to cultural reactions according to the Key to the Species of Propionibacteria (15).

5. Cultural Conditions.

Incubation was carried out at 30°C. Plating was done both aerobically and anaerobically. Anaerobios was obtained through use of the Spray jar.

Details of Method of Purification.

In the first place a solid medium similar to that in which the original culture came was prepared. Inoculations onto slants and into stabs both from stock 25 ml. cultures

and from the original tube were made. No growth was obtained, although some growth was obtained in the same medium without agar.

Gram stains (Hucker modification) of these cultures showed some Gram positive and some Gram negative pleomorphic rods, although the typical morphology as described by Werkman and Brown (15) was not observed.

Since no growth occurred on the above solid medium, the medium of Werkman and Brown (15) was prepared and three Petri dishes and three Spray jars were inoculated from the stock cultures. Growth was obtained both aerobically and anaerobically. Colonies were picked both from Spray jars and Petri dishes into liquid yeast-extract-phosphate medium. About fifty-five such tubes were set up. After incubation for 3 to 4 days a slide was prepared from each tube and fifteen tubes were selected which were most typical morphologically of P.-pentosaceum.

After making transfers from these fifteen cultures to fresh medium, both liquid and solid, the original tubes were titrated for acid production with standard alkali.

The fifteen cultures were then tested for the various cultural reactions according to the Key (15). Nitrate reduction was determined by the method of Randall and Reedy (11). Acid production was determined by adding brom thymol blue to the cultures. The fifteen cultures were also tested for catalase action and for litmus-milk reaction.

The results of these tests are shown in Tables I and II, and from these it may be concluded that culture 40a is a pure culture of Propionibacterium pentosaceum.

TABLE I

CULTURAL REACTIONS OF BACTERIA ON CARBOHYDRATES

Culture Number	Morphology and Gram reaction	Acid Production from: Glucose	Sucrose	Maltose	Xylose	Arabinose	Starch	Dextrin
4	rods, granules, +	0.98	0.30	0.40	-	-	-	-
10	rods, granules +	0.90	0.20	0.60	-	-	-	-
12	rods, granules +	0.80	0.25	-	-	-	-	-
14	rods, granules +	2.50	0.20	0.38	-	-	-	-
15	rods +	1.64	0.50	0.46	-	-	-	-
16	+	1.50	0.20	-	-	-	-	-
20	rods, granules +	1.65	0.38	0.26	-	-	-	-
28	rods, no granules +	2.00	0.30	0.60	-	-	-	-
30	rods, granules +	1.20	0.20	0.69	-	-	-	-
32	rods, +	0.90	0.30	0.94	-	-	-	-
40a	oval rods, +	1.70	0.18	0.90	+	+	-	-
43a	oval rods, +	1.70	-	0.90	+	+	-	-
47	rods, +	2.00	0.46	0.40	-	-	-	-
49	rods, granules +	1.40	0.28	0.48	-	-	-	-
51a	oval rods, granules +	2.20	0.30	0.50	-	-	-	-

Figures represent ml. of 0.1 N acid produced
- No acid produced.

+ Acid produced.

TABLE II

Cultural Reactions
of the
Propionibacteria

Culture Number	Nitrate Reduction	Catalose Action	Litmus-milk Reaction*
4	+	+	Acid
10	-	+	"
12	++	+	"
14	-	+	"
15	-	+	"
16	++	+	"
20	++	+	"
28	+++	+	"
30	+++	+	"
32	+++	+	"
40a	++	-	No Acid
43a	+++	-	"
47	++	+	Acid
49	+++	+	"
51a	+	+	"

---

* No litmus reduction.

2. Chemical Methods.

(a) Alkaline-Diazotized p-aminoacetophenone Method - Prebluda and McCallum (10) introduced the use of alkaline-diazotized p-aminoacetophenone which reacts with the thiazole part of vitamin B_1 to form a purple-red compound which is insoluble in water, but soluble in selective organic solvents, e.g. xylene. The color so extracted can be compared colorimetrically with standards prepared from known thiamine concentrations.

Melnick and Field (9) adapted this reagent to the determination of vitamin B_1 in urine. However, as their method involves a laborious extraction and adsorption procedure, it was attempted to combine the simple adsorption and elution technique of Jowett (6) with the Melnick and Field (8) phenol-ethanol modification of the chemical method using alkaline-diazotized p-aminoacetophenone.

According to this combined procedure the reaction between the thiamine and the reagent is carried out in the same tube, preferably a centrifuge tube with the zeolite. When this was attempted with straight thiamine solutions, however, the purple-red compound seemed to be adsorbed by the zeolite and was not extracted by the xylene. This conclusion was borne out by the fact that when a small amount of Decalso was added to a xylene solution of the purple-red compound, the latter was more or less decolorized and the Decalso assumed a pinkish tinge.

(b) Thiochrome Method. - When solutions of thiamine are treated with alkaline ferricyanide, there is formed the

pale yellow-blue fluorescent oxidation product known as thiochrome. Jansen (5) used this thiochrome test as a method for the quantitative estimation of thiamine with the aid of a fluorometer to measure the fluorescence after the thiochrome was extracted with isobutanol.

Since thenmany modifications of the method have been made for the estimation of vitamin B_1 in biological materials. The main difficulties encountered in the analysis of urine were in suitably concentrating the vitamin and in the elimination of interfering fluorescent substances. These difficulties have been largely overcome by using the synthetic zeolite, Decalso, as an adsorbent. This material has been found to be more specific for thiamine than the adsorbents previously used. (3) (6).

Methods and Materials.

The method used is based essentially on that of Hennessy and Cerecedo (3), with the improvements outlined by Hennessy, (4), and Conner and Straub (1).

It differs in that instead of centrifuge-separatory funnels, the oxidation-extraction step is carried out in 25 ml. glass-stoppered cylinders. These could be centrifuged for separation of the alcoholic layer by protecting the cylinder with a large cork bored to fit over it. Normal butyl alcohol (redistilled)in place of isobutyl alcohol was used to extract the thiochrome formed. Thirty ml. of the butanol was used for the extraction of the thiochrome from the aqueous solution. A smaller volume of the butanol may suffice.

Final clarification of the butanol was obtained by pipetting an aliquot into 0.5 ml. of 95% ethanol in the cuvette in which the fluorescence is to be measured. Conner and Straub (1) used anhydrous Na_2SO_4 in this step after centrifuging and running off the aqueous layer. Fluorescence was measured in a Coleman Electronic Fluorophotometer.

The Fluorophotometer is calibrated by measuring the fluorescence from each of two standard solutions of known thiamine content and expressing as units of solution concentration per meter scale unit.

A standard solution of thiamine (1 ml. = 1.5 micrograms) was prepared in sodium acetate - acetic acid buffer, pH=4.0, with 0.5% chlorobutanol as preservative.

Instrument sensitivity is checked and adjusted before each measurement to give a deflection of around full scale by inserting a stable Reference Solution. For vitamin B_1 this Reference Solution consists of 0.3 mg. of quinine sulfate per 1 litre of N/10 sulfuric acid.

Results:

Calibration of Fluorophotometer

Meter Readings

1st Standard.
0.5 ml. standard B_1 solution
(= 0.75 micrograms B_1) -----37.0 scale units.

2nd Standard.
1.0 ml. standard B_1 solution
(= 1.5 micrograms B_1) -----57.5 " "

Difference in thiamine concentration ----- 0.75micrograms
" " meter readings -----20.5 scale units

Therefore calibration constant, K =

$$\frac{\text{Difference in thiamine concentrations}}{\text{Difference in meter readings.}}$$

$$= \frac{0.75}{20.5}$$

$$= 0.0366 \text{ microgram/scale unit}$$

Estimation of Thiamine in a Solution of Known Concentration.

0.7 ml. of standard solution used, (=1.05 micrograms B_1)

Meter Readings

Test Solution - - - - - - - - - - - - - - 45.0 scale units

Oxidation Blank - - - - - - - - - - - - - 16.0 " "

Difference - - 29.0 " "

Calibration constant = 0.0366 microgram B_1/scale unit.

Therefore, amount of thiamine in test solution =

$$29.0 \times 0.0366 = 1.06 \text{ micrograms.}$$

Estimation of Thiamine in Urine.

When the method was applied to urine, the recovery of thiamine was negligible. The same trouble was encountered by Melnick and Field (9) when they passed urine directly through the adsorption column. They explained the low recovery by the early saturation of the adsorbent with salts from the urine so that further adsorption was impossible.

They overcame the difficulty by extracting the thiamine from concentrated urine with benzyl alcohol and then returning the thiamine to an aqueous solution suitable for adsorption.

SUMMARY

1. Details of culturing Propionibacterium pentosaceum have been worked out, and a pure culture has been obtained.

2. It has been found that the purple-red compound formed by thiamine with alkaline-diazotized p-aminoacetophenone is adsorbed by the synthetic zeolite, Decalso.

3. Thiamine can not be quantitatively adsorbed directly from urine; presumably due to the high concentration of other salts in proportion to thiamine.

PART II

The proper assessment of the physiological and clinical value of any vitamin preparation can only be obtained by biological assay. The investigation reported here is divided into three sections.

Section I deals with the assessment of the relative values of six commercial vitamin B-complex preparations. Section II deals with the effect of supplementing the preparation which was found poorest in Section I, and the comparison of this supplemented preparation with the preparation which was found best in Section I. Section III deals with the comparison of the best preparation with two synthetic preparations, one of equal strength and one of greater strength in several of the factors.

Unfortunately, the work reported in Sections II and III does not cover a time period of 22 weeks as did that in Section I. The experiment is being continued with the aim in mind of making the time periods equal. It is believed, however, that the results are significant enough to be included at this point.

SECTION I

Materials and Methods.

Wistar rats from the laboratory colony were used as the test animals. The rats were taken at weaning, average weight about 40 grams per rat, and divided into groups of ten rats (5 male and 5 female) each. Fifteen such groups were set up. Each group was placed in a separate cage having coarse wire-mesh floors, thus rendering coprophagy difficult.

Each group was weighed thrice weekly for the test period of 22 weeks. These weights were divided by the number of rats in the group at the time of weighing to give the weight per average rat. A protocol of the experiment was kept which included changes in the outward appearance of the rats, dates on which casualties were found, and autopsy reports. Autopsies were performed on all casualties except a few which occurred during the night and were mostly eaten before they were found the next morning. Autopsies were confined to gross examination of the viscera. Specimens of the liver were kept in all cases and occasionally of other structures, such as adrenals, kidneys, lungs, spleen, as well. All specimens were labelled and preserved in 10% formalin. Each rat was fed every day the designated amount of B-complex preparation. They all had water and B-free diet ad lib.

The vitamin B-complex free diet (Smaco) has the following composition: sucrose 68%, vitamin-free casein 18%, vegetable oil 8%, U.S.P. salt mixture No.2 4%, U.S.P. cod liver oil 2%. The salt mixture consists of sodium chloride 1.73 gm., magnesium sulfate 5.45 gm., sodium acid phosphate 3.47 gm., potassium acid phosphate 9.54 gm., calcium acid phosphate 5.40 gm., ferric citrate 1.18 gm. and calcium lactate 13.00 gm. The sodium chloride content of the diet was 0.174%.

Six commercial preparations of the vitamin B-complex, purchased in a drug store were used. The relative composition of these according to label information are shown in Table III on the basis of 1 c.c. or 1 tablet. This table also shows the

doses recommended and average daily human requirements of these vitamins. The manufacturer, trade name, form and source of these preparations were as follows:-

A - Abbott: "Vitamin B Complex", syrup: yeast.

B - Ayerst, McKenna and Harrison: "Beminal", tablets, yeast.

C - Lederle: "Vitamin B Complex, Oral", syrup, liver.

D - Mowatt and Moore: "Vitamin 'B' Complex", tablets, yeast.

E - Parke-Davis: "Abdol", capsules, liver.

F - Wyeth: "B-Plex", elixir, rice-bran.

Solutions of each of the six preparations were prepared so as to contain 4 I.U. vitamin B_1 per 0.1 ml. Details of the preparation of these solutions as well as of the actual method of feeding the rats are given in the Appendix.

TABLE III

SHOWING THE RELATIVE COMPOSITIONS OF THE SIX COMMERCIAL PREPARATIONS USED, THE DOSES RECOMMENDED ON THE LABELS, COMPARED WITH AVERAGE HUMAN REQUIREMENTS DAILY

	MICROGRAMS PER 1 C.C. OR 1 TABLET OF					
PREPARATION	THIAMINE	RIBOFLAVIN	PYRIDOXINE	PANTOTHENIC ACID	NICOTINIC ACID	DOSE RECOMMENDED ON LABEL
A - 1 c.c.	300	120	50	*100	1200	8 c.c. - Adults
B - 1 tablet	3300	150	150	*275	8370	3 tablets/day
C - 1 c.c.	375	150	50	280	*1437	8 c.c. - Adults
D - 1 tablet	1500	500	100	184	4000	3 tablets/day
E - 1 capsule	600	100	+	+	+	1 capsule/day
F - 1 c.c.	125	100	50	130	625	Only as per prescription (8c.c.)
Average Daily Requirement	1500	1500	750?	1500?	20,000	

1 I.U. of Thiamine = 3 micrograms (0.003 mg.)

1 Sherman-Bourquin Unit of Riboflavin = 2.5 micrograms

* Not stated on label.

Plan of the Experiment.

As thiamine itself has received considerable attention already it was felt that more could be learned concerning the effects of the other factors of the B-complex by feeding the preparations on an equal thiamine basis. Four I.U. of thiamine per day is considered adequate for the rat, so this was taken as the dose-level for the first six groups of rats. Since additional differences in the effect of other members of the B-complex might be shown up if the thiamine intake were inadequate, a further six groups of rats were started on a daily intake of two I.U. B_1 per rat, and a further three groups on only one I.U. B_1 per rat.

Results.

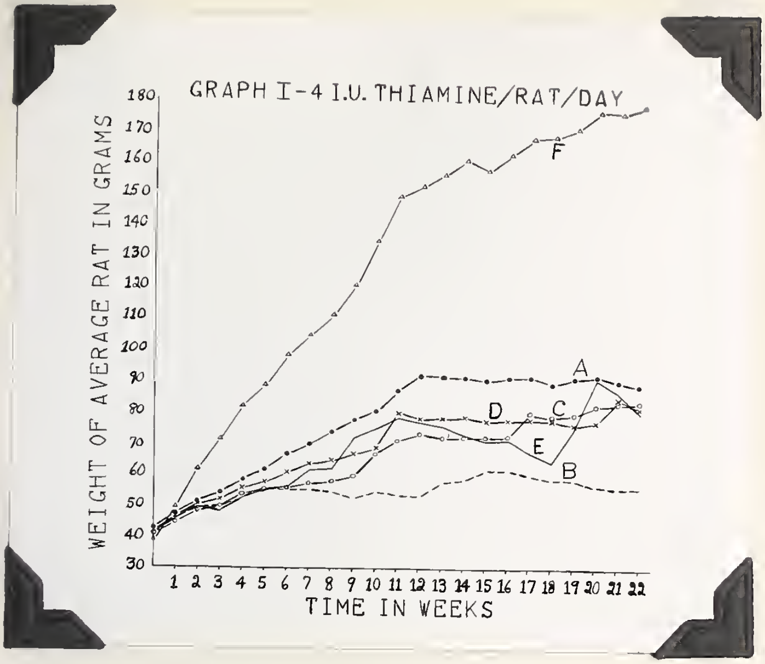
The results are shown in three ways.

(1). As a comparison of the growth curves, and the growth obtained, Graphs I, II and III;

(2). As a comparison of the growth rates, that is, the total gain in weight divided by the 22 weeks of the experiment, Table IV;

(3). As a comparison of the number of rats that survived, Table IV.

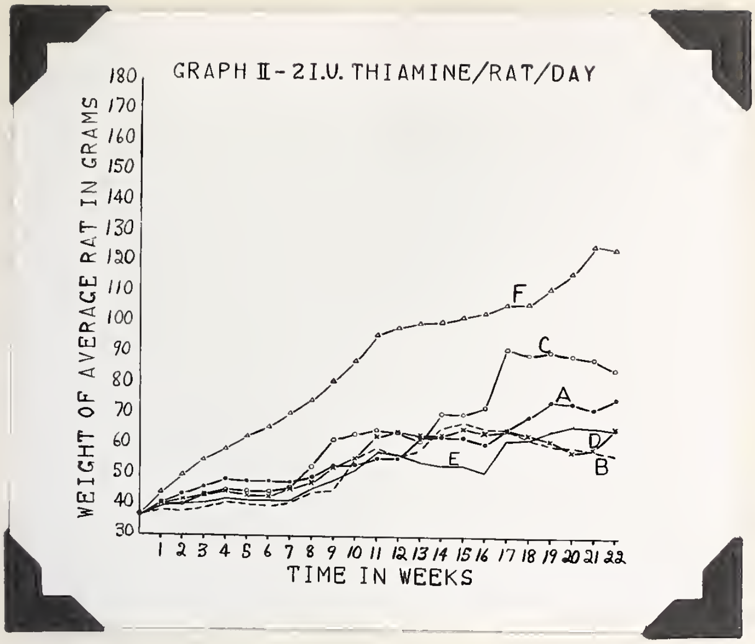
Graph I shows the average growth curves obtained when the six B-complex preparations were fed so that each rat received 4 I.U. of thiamine daily. The differences are then due to the other members of the B-complex which vary in relative amounts in each of the preparations.



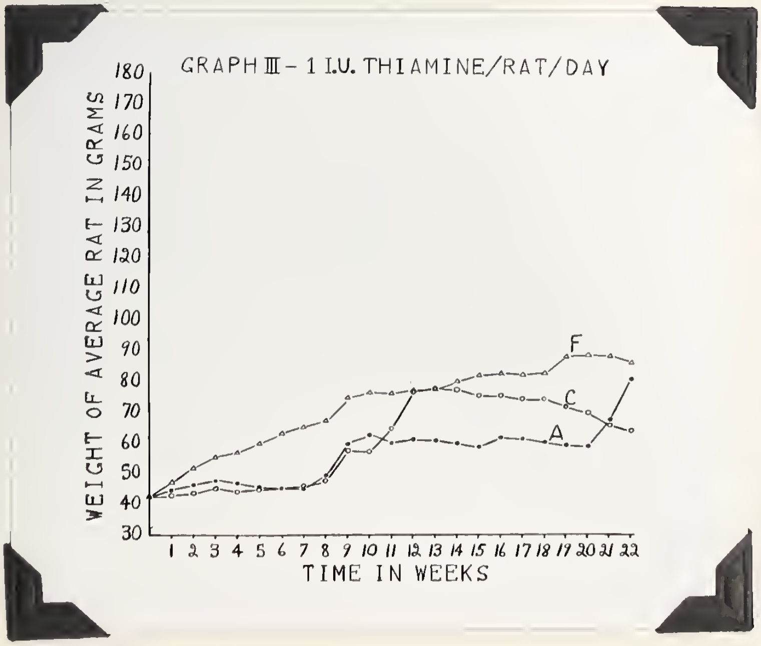
Note.

The decline at 15 weeks in the growth curve for Preparation F in this graph was due to the termination of a pregnancy that had developed. Both male and female rats had been confined to the same cage until the pregnancy was first noticed, when they were separated. Such a happening did not occur in any of the other 14 groups although no separation of the sexes was made.

Graph II shows the average growth curves when each rat received 2 I:U: thiamine daily. In this case the rats were receiving a sub-optimal amount of thiamine, and by putting them in a critical condition, other differences due to the other components of the B-complex are brought out.



Graph III shows the average growth curves obtained when each rat received only 1 I.U. thiamine daily.



Abrupt rises in the growth curves in the three graphs are generally due to the deaths of rats that were of lower weight than the other rats of the same group. This has been emphasized also when more than one rat died at or closely to the same time. An example of the exception to this is shown by the curve A of Graph I, where although casualties occurred, the curve is quite continuous. This curve also shows very well the "plateau" effect obtained when the components of the B_2-complex are received in suboptimal amounts.

A better comparison may be made from a consideration of the rate of gain in weight over the 22 weeks' period of the experiment and the number of rats that survived, in comparison to the actual amounts of the various B-complex components that each rat received. This information is given in Table IV.

The general condition of the rats and the number of survivors in each group are shown in photographs in the Appendix.

TABLE IV

COMPARISON OF GROWTH RATES WITH THE AMOUNTS OF VITAMIN B-COMPLEX FACTORS RECEIVED

GROUP NO.	PREPARATION	MICROGRAMS RECEIVED PER RAT PER DAY OF:					RESULTS AFTER 22 WEEKS	
		THIAMINE	RIBOFLAVIN	PYRIDOXINE	PANTOTHENIC ACID	NICOTINIC ACID	RATE OF GAIN gms/wk.	SURVIVORS OUT OF 10
1 - 4	A	12.0 (4 I.U.)	4.8	2.0	4.0[1]	48.0	2.17	6
2 - 4	B	12.0	0.54	0.54	1.0[1]	30.4	0.68	1
3 - 4	C	12.0	4.8	1.6	9.0	46.0[1]	1.95	5
4 - 4	D	12.0	4.0	0.8	1.5	32.0	1.84	3
5 - 4	E	12.0	2.0	+	+	+	1.79	2
6 - 4	F	12.0	9.6	4.8	12.5	60.0	6.39	9[2]
1 - 2	A	6.0	2.4	1.0	2.0[1]	24.0	1.76	2
2 - 2	B	6.0	0.27	0.27	0.5[1]	15.2	0.91	2
3 - 2	C	6.0	2.4	0.8	4.5	23.0[1]	2.19	2
4 - 2	D	6.0	2.0	0.4	0.75	16.0	1.35	1
5 - 2	E	6.0	1.0	+	+	+	1.30	1
6 - 2	F	6.0	4.8	2.4	6.2	30.0	3.97	6
1 - 1	A	3.0	1.2	0.5	1.0[1]	12.0	1.76	1
3 - 1	C	3.0	1.2	0.4	2.2	11.5[1]	0.99	1
6 - 1	F	3.0	2.4	1.2	3.1	15.0	1.99	7

[1] Calculated.

[2] One death from congenital defect.

Discussion.

From the graphs and Table IV it is seen at once that one commercial preparation of the vitamin B-complex (F) has given outstandingly better results than any of the others. Since the natural source of this preparation was rice-bran it might be assumed that the difference was due to this fact. However, this was the only rice-bran preparation assayed and besides it is known to have been enriched with added thiamine and riboflavin.

Of the other preparations, A was best and B poorest in this test.

It will also be seen by comparing Graphs I and II, that reducing the thiamine intake to a suboptimal level has produced a change in the relative positions of the six preparations.

More precise comparisons may be made from Table IV, although it is difficult to show that differences in any one factor account for the differences obtained. Considering only the first set of six groups in which each rat received 4 I.U. thiamine daily, the difference in rate of gain and the number of survivors closely parallels the amount of riboflavin received. This correlation is better than can be made with any of the other components of the B-complex, or with several combinations of the other components. It is not pyridoxine or pantothenic acid in view of the results with preparation A, C and D where the rate of gain is not in proportion to the amounts of either of the two vitamins received. Nor is it due to nicotinic acid when the results of A and C, or of B and D are compared with the amounts of nicotinic acid received in each case. It may

be assumed therefore, from these figures, that the differences among the various preparations shown in Graph I, are due to differences in riboflavin content.

That riboflavin content is not the final answer is apparent from Graph II and the second lot of six groups in Table IV in which each rat received 2I.U. thiamine per day. It is clear that some other factor or factors are playing a part both on survival and on growth rate. For instance, Preparation D was supplying twice as much riboflavin as E, yet the average gain was practically the same in each case. Again preparation F was supplying twice as much riboflavin as either A or C, yet the average gains are not in proportion, that of A being as much less than one-half the gain with F as that of C is greater. The important factor is not nicotinic acid either in view of the same three results. There remain pyridoxine and pantothenic acid and less known components. In the case of pyridoxine, although Preparation A was supplying more of this factor than Preparation C, the average gain was less. In the case of Pantothenic acid, the various preparations can certainly be arranged in the order of increasing pantothenic acid content, but the average gains are not in proportion to the amounts of that factor received. Consideration of the bottom set of three groups shows an average gain definitely not in proportion to the ratios of pantothenic acid nor to any of the other components with the possible exception of nicotinic acid, which, however, was previously eliminated. While comparison is difficult, the results show that at different levels of thiamine intake, the importance of other components of the B-complex

may vary. This conclusion can be seen more clearly from a consideration of Table V in which the growth rates have been grouped together for convenience in comparison. There have been included in this Table also certain calculated growth rates which are what would be expected if decrease in the amount of B-complex received resulted in a proportional decrease in the growth rate.

It will be seen at once that the actual growth rates obtained on the 2 I.U. thiamine/day and on the 1 I.U. thiamine/day levels (columns 2 and 4) are greater than what might be expected (columns 3 and 5) if dilution were the sole cause. Also, as stated before, the order of superiority of the six preparations on the 2 I.U. thiamine/day level is different from that found on the 4 I.U. thiamine/day level. It is interesting to note, too, that for preparation A, the actual rate of gain on the 1 I.U. thiamine/day level is exactly the same as that obtained on the 2 I.U. thiamine/day level, while for preparation F, the actual rate of gain on the former level is exactly one-half of that obtained on the latter one. It would seem, therefore, that in the first instance dilution of the amount of vitamins received had no effect, while in the second it was the sole influence.

TABLE V

COMPARISON OF THE GROWTH RATES OBTAINED WITH CERTAIN CALCULATED ONES.

Group No., Preparation	Actual Rate of*Gain on 4 I.U.B_1/day	Actual Rate of Gain on 2 I.U.B_1/day	Rate of Gain Calculated as 1/2 that on 4 I.U.	Actual Rate of Gain on 1 I.U.B_1/day	Rate of Gain Calculated as 1/4 that on 4 I.U.	Rate of Gain Calculated as 1/2 on 2 I.U.
1 (A)	2.17	1.76	1.09	1.76	0.54	0.88
2 (B)	0.68	0.91	0.34			
3 (C)	1.95	2.19	0.98	0.99	0.49	1.10
4 (D)	1.84	1.35	0.92			
5 (E)	1.79	1.30	0.90			
6 (F)	6.39	3.97	3.20	1.99	1.60	1.99

* Rate of gain expressed in gms./week.

The arbitrary choice of thiamine as the basis of feeding the different preparations places at a disadvantage a preparation such as B, containing large amounts of thiamine. Had the preparations been fed, for example, on an equal riboflavin basis, the results would likely have been different. The results, therefore, do not warrant conclusions about the relative physiological values of the preparations, except on an equal thiamine basis.

The results show though that a suitable ratio of components is of prime importance, since equal thiamine intake was not good enough in the presence of unequal amounts of other components.

Summary.

1. Six commercial preparations of the vitamin B-complex have been compared by feeding them as supplements to groups of rats on a vitamin B-complex-free diet, so that the thiamine intake was uniform in all groups.
2. The best results came from using a preparation (F) having the following proportion among known members of the vitamin B-complex: Thiamine 1.0, Riboflavin 0.8, Pyridoxine 0.4, Pantothenic Acid 1.0, Nicotinic Acid 5.0.
3. The poorest results were obtained using a preparation (B) having the following proportion among the components: Thiamine 1.0, Riboflavin 0.05, Pyridoxine 0.05, Pantothenic Acid 0.1, Nicotinic Acid 2.5.
4. These results emphasize the importance of having adequate amounts of all the B_2-complex along with thiamine. The gross disproportion among these components observed

in some commercial preparations apparently resulted from adding such synthetics as thiamine or nicotinic acid to the original source material.

5. At different levels of thiamine intake, the relative importance of the components of the B_2-complex changed. Thus, with higher amounts of thiamine, the riboflavin content was of greatest importance, whilst with smaller quantities of thiamine, the pyridoxine and nicotinic acid contents seemed to be more important than that of riboflavin.

SECTION II

Methods and Materials.

These were similar to those described under Section I with the exception of the supplements fed in addition to the B-free diet. The supplements used were vitamin B-complex preparations B and F as in Section I, and preparation B supplemented with various factors. The composition of each of these supplements is given in Table VI. Details of their preparation are given in the Appendix.

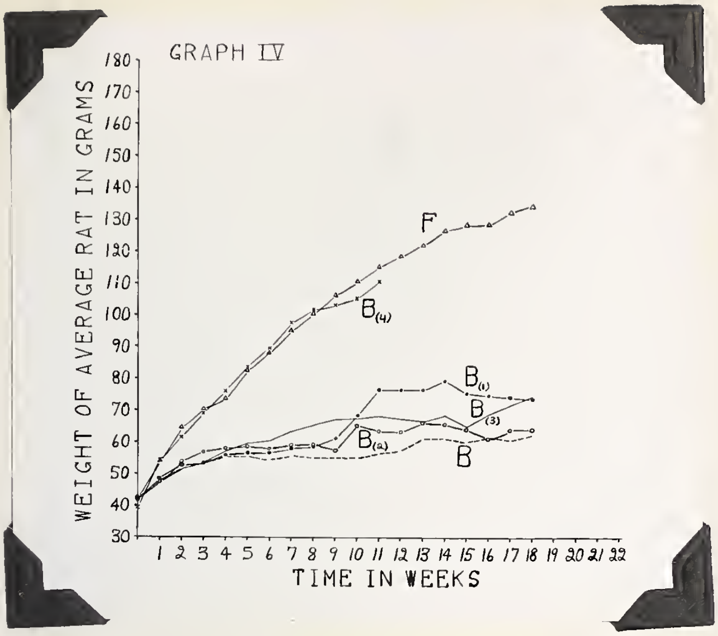
Each rat was fed every day as before, the daily thiamine intake per rat being 4 I.U.

Purpose of Experiment.

To disprove or confirm the conclusion reached in Section I that a suitable ratio of all components of the B-complex is necessary for growth; and to determine whether two of the natural sources of the B-complex, viz. yeast and rice-bran, show any difference if the concentrations of the known components are the same in both cases.

Results.

The results are presented as in Section I. Graph IV shows the growth curves and growth obtained; Table VI shows the growth rates and survivors at 11 and 18 weeks, compared with the actual intake of each component of the B-complex.



Discussion.

It will be seen at once from Graph IV that neither riboflavin, pyridoxine nor pantothenic acid alone were sufficient to bring preparation B up to the standard of F. But when all the factors were added, preparation $B_{(4)}$, there was no significant difference between that and preparation F as far as the experiment has gone. However, it appears from the graph that as the experiment continues, less known factors, i.e. the natural source of the B-complex, may play a part, since there is a tendency for the curve for preparation $B_{(4)}$ to fall below that of F.

From the results in the Table VI it is observed that increasing the concentration of any or all the factors, increases the growth rates, but not to the same degree. However, such a result is to be expected. It is also apparent that pantothenic acid plays an important part in survival. Indeed it was not until 16 weeks, that the first and so far, only casualties in

TABLE VI

COMPARISON OF GROWTH RATES WITH PREPARATIONS B, F, AND SUPPLEMENTED B.

Group and Preparation	Micrograms Received per Rat per Day of:					Results after 11 weeks		Results after 18 weeks	
	Thiamine	Riboflavin	Pyridoxine	Pantothenic Acid	Nicotinic Acid	Rate of Gain*	Survivors Out of 10	Rate of Gain*	Survivors Out of 10
1 - B	12.0	0.54	0.54	1.0	30.4	1.27	8	1.12	5
2 - B$_{(1)}$	4 I.U. 12.0	9.6	0.54	1.0	30.4	3.15	6	1.77	6
3 - B$_{(2)}$	12.0	0.54	4.0	1.0	30.4	1.94	6	1.21	3
4 - B$_{(3)}$	12.0	0.54	0.54	13.9	30.4	2.38	10	1.79	6
5 - B$_{(4)}$	12.0	9.6	4.8	13.5	60.0	6.45	10	--	-
6 - F	12.0	9.6	4.8	12.5	60.0	6.65	10	5.12	10

* Rate of Gain Expressed in gm/wk.

that group (no.4) receiving added pantothenic acid, occurred. The first casualty in group 3 (added pyridoxine) occurred at 7 weeks, the first in groups 1 and 2 (added riboflavin) at 9 weeks each.

SUMMARY

1. Six preparations of the vitamin B-complex have been compared as in Section I. Two of these preparations were the same as in Section I. The other four consisted of one of the former preparations supplemented separately with riboflavin, pyridoxine, and pantothenic acid, and with these three factors and nicotinic acid together.

2. The results show the definite necessity of adequate amounts of all components of the B-complex in any preparation.

3. The results show also that only over long periods of time is there liable to be any significant difference between yeast and rice-bran as sources of the B-complex, provided the concentrations of the known components are equal in both cases.

SECTION III

Materials and Methods.

These were similar to those described under Section I with the exception of the supplements fed in addition to the B-free diet. The supplements used were vitamin B-complex preparation F and two solutions of synthetic B-complex factors.

The composition of each supplement is given in Table VII. Details of their preparation are given in the Appendix.

Each rat was fed a daily dose containing 4 I.U. thiamine.

Purpose of Experiment.

To determine how well synthetic factors alone, would support growth; and whether there was a harmful imbalance among the various components when the riboflavin and pyridoxine contents had been increased.

Results.

The results are presented as in the two previous sections. Graph V shows the growth curves and the growth obtained. Table VII shows the growth rates and survivors at 11 and 18 weeks, compared with the actual intake of each component of the B-complex.

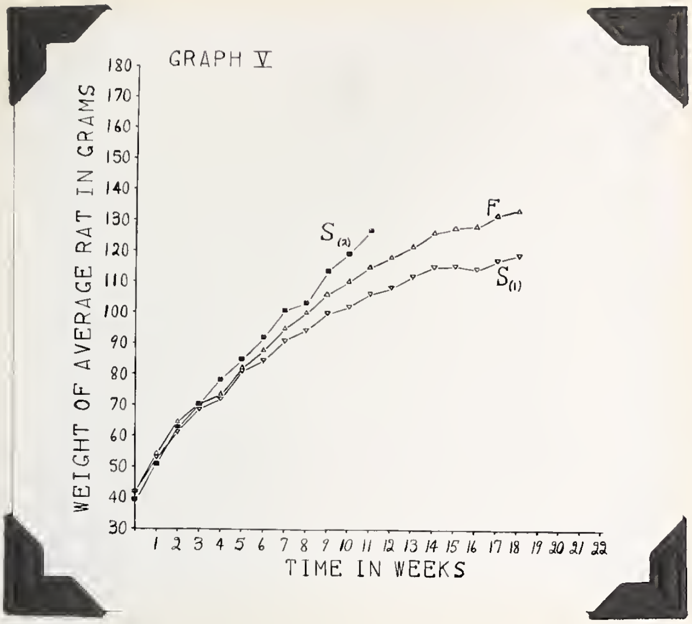
TABLE VII

COMPARISON OF GROWTH RATES WITH A NATURAL PREPARATION F AND TWO SYNTHETIC ONES, $S_{(1)}$ AND $S_{(2)}$.

Group and Preparation	Micrograms Received per Rat per Day of:					Results after 11 weeks		Results after 18 weeks	
	Thiamine	Riboflavin	Pyridoxine	Pantothenic Acid	Nicotinic Acid	Rate of Gain*	Survivors Out of 10	Rate of Gain*	Survivors Out of 10
1[1] - F	12.0	9.6	4.8	12.5	60.0	6.65	10	5.12	10
2 - $S_{(1)}$	12.0	9.6	4.0	12.6	60.0	5.90	10	4.31	10
3 - $S_{(2)}$	12.0	38.4	8.6	12.5	60.0	8.00	9	--	-

* Rate of Gain expressed in gm/wk.

[1] This group is the same as group 6 of Table VI, Section II.



Discussion:

Both Graph V and Table VII show that the synthetic preparation $S_{(1)}$, approximately equal to preparation F, did not give as good results as the latter, while the other synthetic preparation, $S_{(2)}$, gave better results than F. The low results with $S_{(1)}$ is probably entirely due to the lack of the less known components of the B-complex such as biotin and inositol, and which are present in the rice-bran base of preparation F. However, since $S_{(1)}$ was a little lower than F in pyridoxine content, it should fall below F on the graph unless the difference were indetectable by this method of assay. The good result with preparation $S_{(2)}$ shows the beneficial effect of high riboflavin and pyridoxine contents on growth. Whether the effect will also be to reduce the number of survivors remains to be seen.

SUMMARY

1. A natural preparation of the vitamin B-complex has been compared to two synthetic ones by means similar to those used in Section I.

2. The results show the benefit of including in any preparation a natural source of the B-complex to supply the less known components. The rice bran base of preparation F is shown to be a good source of these components.

APPENDIX

APPENDIX

1.

DETAILS OF PREPARATION OF SUPPLEMENTS FED IN SECTION I. AND METHOD OF FEEDING.

A. One hundred ml. of the commercial preparation were pipetted into a 250 ml. volumetric flask and diluted to volume with distilled water.

B. Nine and one-eleventh tablets were placed in a volumetric flask similar to above, with a little distilled water. When the tablets had disintegrated, the flask was filled to volume and well shaken. The solution was then filtered. It was found that filtration was facilitated by a preliminary centrifugation of the solution.

The 1/11 tablet was taken as 1/11 of the weight of a tablet finely powdered.

C. Eighty ml. of original syrup measured out in a graduated cylinder were washed into a 250 ml. volumetric flask and diluted to volume. The final solution was filtered.

D. Twenty tablets were placed in a 250-ml. flask and made up as in case of preparation C.

E. These capsules also contained vitamins A and D. The contents of 50 capsules were well shaken with water and the emulsified fatty material filtered off. The filtrate was made up to 250 ml.

F. Two hundred and fifty ml. of the original syrup were measured out in a volumetric flask. Ten ml. were withdrawn by means of a pipette and the remainder made to volume with water.

A few drops of chloroform were added to all preparations except F, which contained approximately 16% alcohol and didn't require any further preservative. All 6 stock solutions were stored at refrigeration temperature. These solutions were conveniently diluted to contain 2 I.U. B_1 per 0.1 ml. and 1 I.U.B_1 per 0.1 ml. as required. Ten ml. of each of the 15 solutions were kept in small Erlenmeyer flasks and from these the day's rations were withdrawn by suction into the feeding burette. All solutions were shaken before the aliquot was selected. These flasks were also stored in the refrigerator between use. The feeding burette was a 1 ml. pipette graduated in 0.01 ml. divisions, clamped upright in a stand. A length of rubber tubing from the top of the pipette allowed the solution to be sucked up to the zero mark. A delivery tip was connected to the tip of the pipette by means of a short length of thin-walled rubber tubing. The delivery tip was drawn out from thick-walled, wide-bore capillary tubing. The tip was fire-polished and its walls were thick enough so that they would not be crushed by the jaws of the rats. A small pinchcock just above the tip was used to regulate the flow. This arrangement allowed considerable freedom of movement in the actual feeding. Each rat was fed 0.1 ml. of its designated solution. The error in running out this amount was usually well below 0.005 ml. Each dose was introduced well back

in the mouth of the rat so that it had to be swallowed. This was most important with those solutions which the rats found unpalatable. One filling of the burette was thus sufficient for each group of ten rats. Solutions of each preparation were introduced into the burette progressively from the more dilute to the more concentrated one. Between solutions of different preparations the burette was well rinsed with distilled water and drained as much as possible.

2.

DETAILS OF PREPARATION OF SUPPLEMENTS FED IN SECTION II.

B. Three and seven-elevenths "Beminal" tablets made up to 100 ml. volume as described under Appendix 1.

$B_{(1)}$ Three and seven-elevenths tablets + 9.1 mg. riboflavin made up to 100 ml. as above. Riboflavin content of final solution equal to 9.6 micrograms per 0.1 ml.

$B_{(2)}$ Three and seven-elevenths tablets + 0.047 ml. of a pyridoxine hydrochloride solution (Squibb parenteral, 1 ml. = 100 mg.) amd made to 100 ml. as above. Final solution equal to 4.8 micrograms pyridoxine hydrochloride per 0.1 ml., or 4.0 micrograms free pyridoxine per 0.1 ml.

$B_{(3)}$ Three and seven-elevenths tablets + 0.28 ml. of a calcium pantothenate solution (Squibb parenteral, 1 ml. = 50 mg. Ca Pantothenate = 46 mg. pantothenic acid) and made to 100 ml. as above. Pantothenic acid content of final solution equal to 13.9 micrograms per 0.1 ml. This figure includes the

amount which was later calculated to be present in the original tablets.

$B_{(4)}$ Three and seven-elevenths tablets + 9.1 mgm. riboflavin + 4.3 mgm. pyridoxine + 12.5 mgm. Pantothenic acid + 29.6 mgm. nicotinic acid, all made up to 100 ml. with distilled water as above.

F. Ninety-six ml. of "B-Plex" solution diluted to 100 ml.

All solutions were preserved as before at refrigeration temperature. Small quantities were kept apart for day to day use also as before.

3.

DETAILS OF PREPARATION OF SUPPLEMENTS FED IN SECTION III.

F. Similar to Solution F in Section II.

$S_{(1)}$. These quantities of the synthetic factors were dissolved in 16% alcohol and made to 200 ml.: thiamine 24.0 mg. riboflavin 19.2 mg., Pyridoxine hydrochloride 9.6 mg., calcium pantothenate 27.2 mg. (due to miscalculation this amount should have been 26.9 mg. instead of 27.2 mg.), nicotinic acid 120.0 mg. Again due to the error of calculating vitamin B_6 as pyridoxine hydrochloride instead of the free pyridoxine, the final concentration of the latter was only 4.0 micrograms per 0.1 ml.

$S_{(2)}$ These quantities of the synthetic factors were dissolved in 16% alcohol and made up to 200 ml.: thiamine 24.0 mg., riboflavin 76.8 mg., pyridoxine hydrochloride 23.3 mg., calcium pantothenate 26.9 mg., nicotinic acid 120.0 mg. The riboflavin did not dissolve completely but remained to some extent as a suspension. Four drops of 85% phosphoric acid were added in an attempt to dissolve or stabilize this suspension but with no success. The solution was well shaken before the daily dose was withdrawn, and no appreciable settling occurred in the feeding burette during the time of feeding (about one minute).

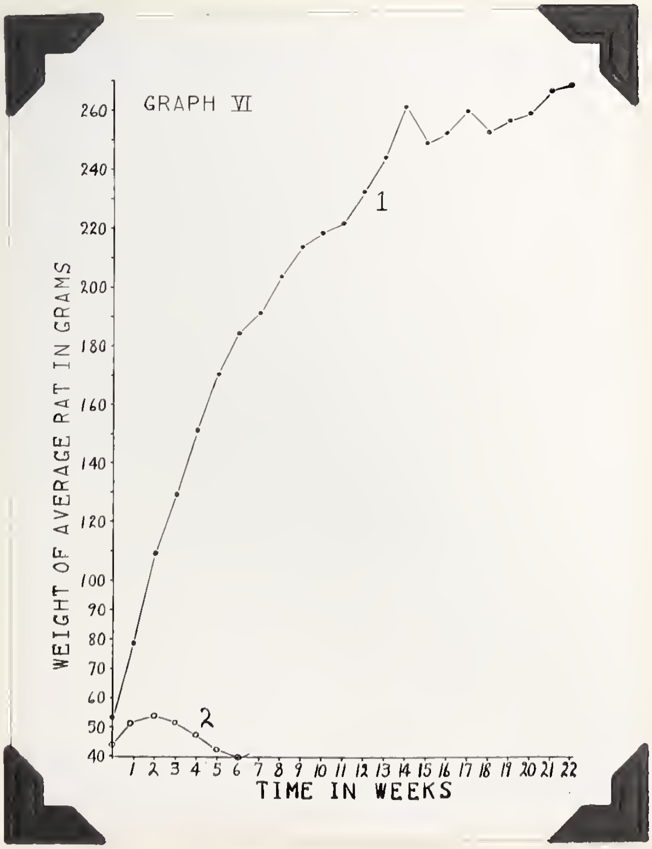
All solutions were stored in the refrigerator.

4.

GROWTH OF RATS ON A COMPLETE DIET AND ON A VITAMIN B-COMPLEX-FREE DIET.

The results of feeding a complete diet ("Checkers") to a group of 10 rats (5 male and 5 female) are shown by Curve 1 on Graph VI. The discontinuity of the curve near the end resulted from breeding when one of the males got in with the females.

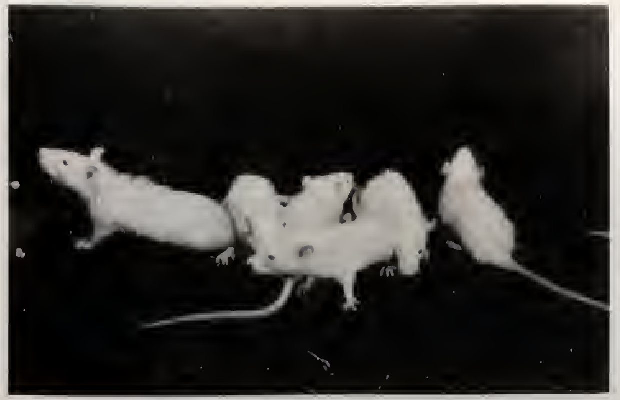
The results of feeding a B-free diet (Smaco) to a similar group of rats are shown by Curve 2.



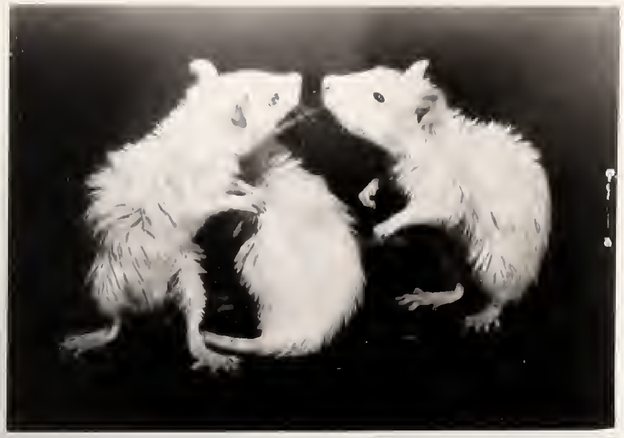
5.

PHOTOGRAPHS OF SURVIVORS FROM GROUPS IN SECTION I AT 22 WEEKS.

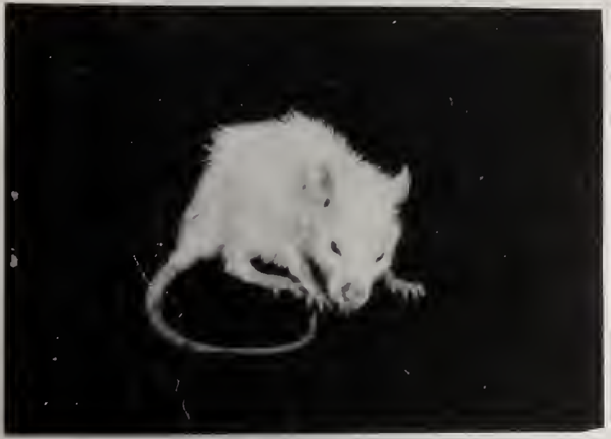
SURVIVORS OF SIX GROUPS ON
DAILY THIAMINE INTAKE
OF 4 I.U.



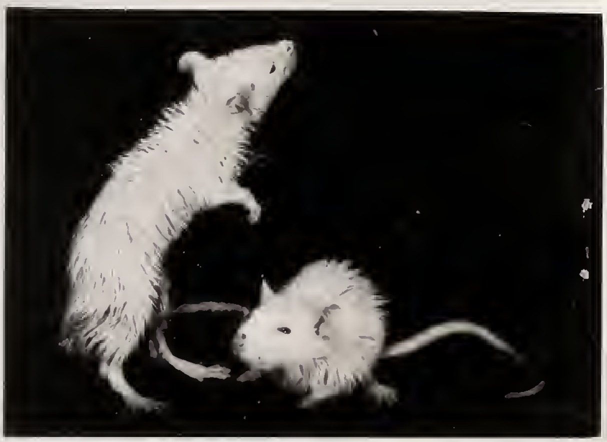
Survivors of 10 rats on
product A at 22 weeks.



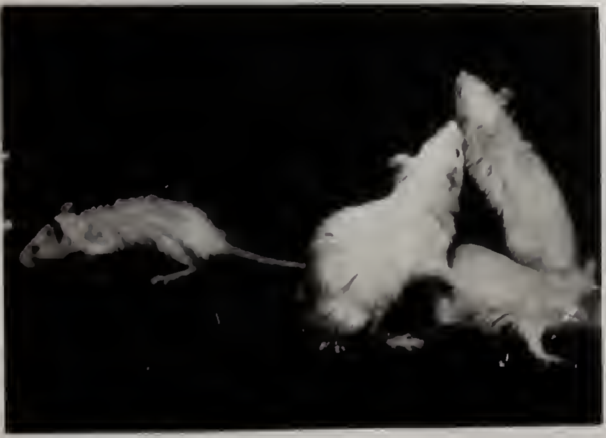
Survivors of 10 rats on
product D at 22 weeks.



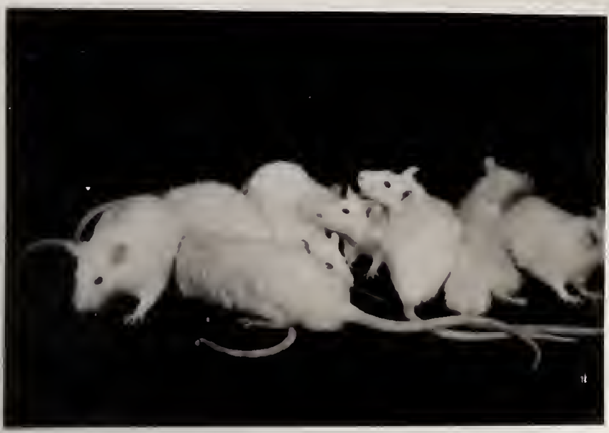
Survivors of 10 rats on
product B at 22 weeks.



Survivors of 10 rats on
product E at 22 weeks.



Survivors of 10 rats on
product C at 22 weeks.



Survivors of 10 rats on
product F at 22 weeks.

LEW. F. CLARKE
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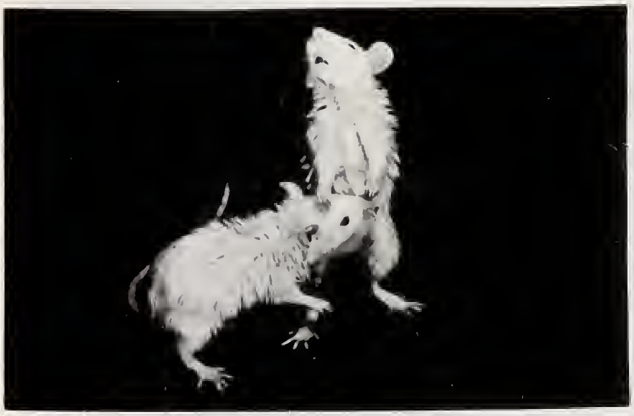
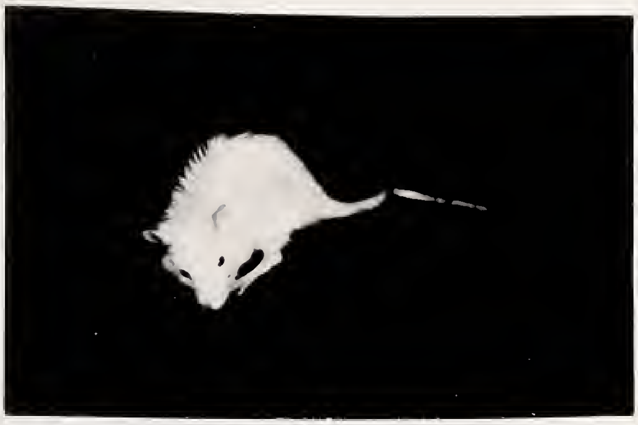
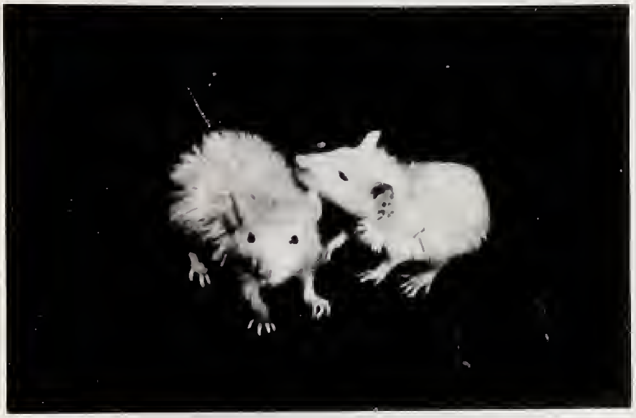
SURVIVORS OF SIX GROUPS ON
DAILY THIAMINE INTAKE
OF 2 I.U.



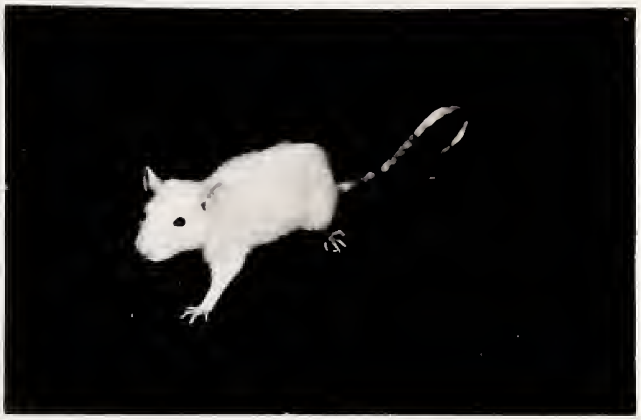
Survivors of 10 rats on
product A at 22 weeks.



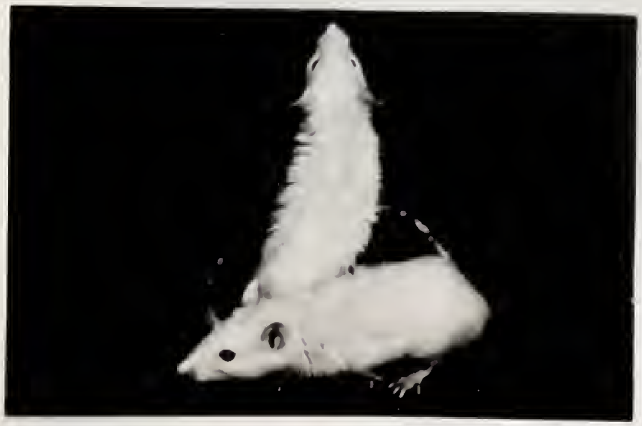
Survivors of 10 rats on
product D at 22 weeks.



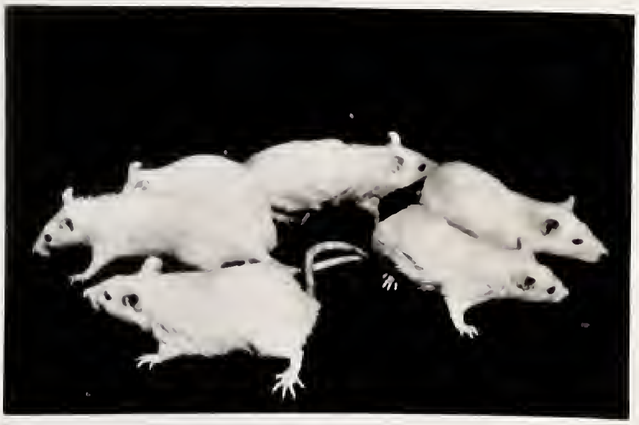
Survivors of 10 rats on
product B at 22 weeks.



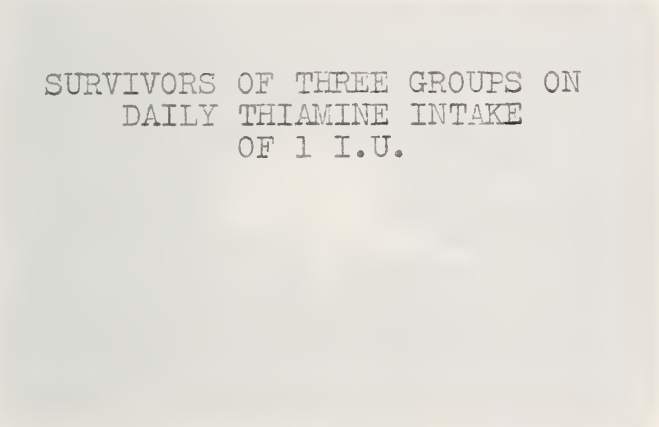
Survivors of 10 rats on
product E at 22 weeks.



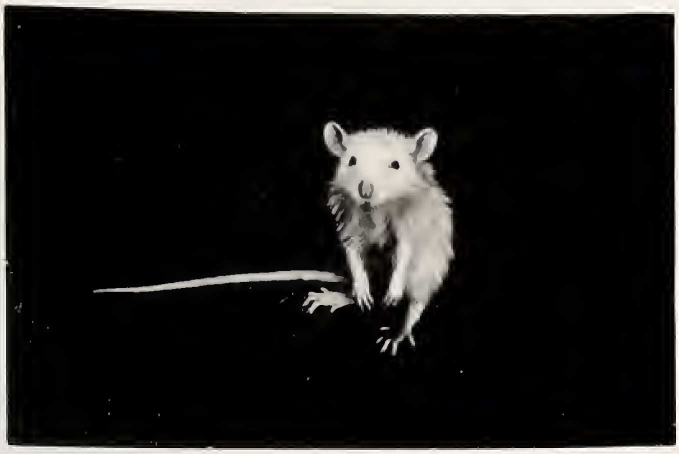
Survivors of 10 rats on
product C at 22 weeks.



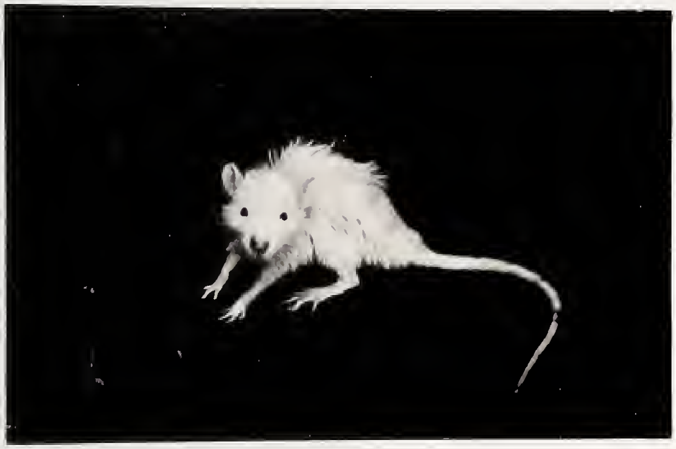
Survivors of 10 rats on
product F at 22 weeks.



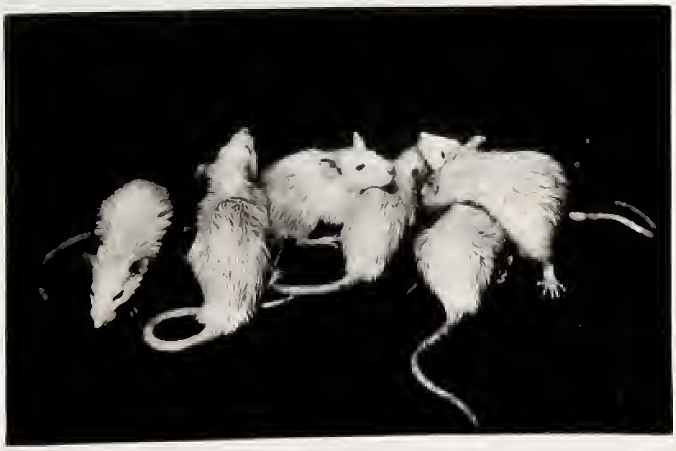
SURVIVORS OF THREE GROUPS ON
DAILY THIAMINE INTAKE
OF 1 I.U.



Survivors of 10 rats on
Product A at 22 weeks.



Survivors of 10 rats on
Product [illegible] at 22 weeks.



Survivors of 10 rats on
Product [illegible] at [illegible] weeks.

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